Stem Cell Research 15 (2015) 403–418

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Characterization of DLK1(PREF1)$^+$/CD34$^+$ cells in vascular stroma of human white adipose tissue

Marit E. Zwierzina [a,1], Asim Ejaz [b,1], Mario Bitsche [a], Michael J.F. Blumer [a], Maria C. Mitterberger [b], Monika Mattesich [c], Arno Amann [d], Andreas Kaiser [b], Elisabeth J. Pechriggl [a], Susanne Hörl [b], Ursula Rostek [b], Gerhard Pierer [c], Helga Fritsch [a], Werner Zwerschke [b,*]

[a] Division for Clinical and Functional Anatomy, Department for Anatomy, Histology and Embryology, Medical University of Innsbruck, Austria
[b] Cell Metabolism and Differentiation Research Group, Institute for Biomedical Aging Research, University of Innsbruck, Austria
[c] Department of Plastic, Reconstructive and Aesthetic Surgery, Medical University of Innsbruck, Austria
[d] Department of Internal Medicine V, Medical University of Innsbruck, Austria

ARTICLE INFO

Article history:
Received 4 April 2014
Received in revised form 7 August 2015
Accepted 13 August 2015
Available online 15 August 2015

Keywords:
Adipogenesis
Adipose-derived stromal/progenitor cells
α-SMA
CD34
CD90
Clonal expansion
DLK1
Vascular niche

ABSTRACT

Sorting of native (unpermeabilized) SVF-cells from human subcutaneous (s)WAT for cell surface staining (cs) of DLK1 and CD34 identified three main populations: ~10% stained cs-DLK1$^+$/cs-CD34$^-$, ~20% cs-DLK1$^+$/cs-CD34$^{+\,dim}$ and ~45% cs-DLK1$^-$/cs-CD34$^+$. FACS analysis after permeabilization showed that all these cells stained positive for intracellular DLK1, while CD34 was undetectable in cs-DLK1$^+$/cs-CD34$^-$ cells. Permeabilized cs-DLK1$^-$/cs-CD34$^+$ cells were positive for the pericyte marker α-SMA and the mesenchymal markers CD90 and CD105, albeit CD105 staining was dim (cs-DLK1$^-$/cs-CD34$^+$/CD90$^+$/CD105$^{+\,dim}$/α-SMA$^+$/CD45$^-$/CD31$^-$). Only these cells showed proliferative and adipogenic capacity. Cs-DLK1$^+$/cs-CD34$^-$ and cs-DLK1$^+$/cs-CD34$^{+\,dim}$ cells were also α-SMA$^+$ but expressed CD31, had a mixed hematopoietic and mesenchymal phenotype, and could neither proliferate nor differentiate into adipocytes. Histological analysis of sWAT detected DLK1$^+$/CD34$^+$ and DLK1$^+$/CD90$^+$ cells mainly in the outer ring of vessel-associated stroma and at capillaries. DLK1$^+$/α-SMA$^+$ cells were localized in the CD34$^-$ perivascular ring and in adventitial vascular stroma. All these DLK1$^+$ cells possess a spindle-shaped morphology with extremely long processes. DLK1$^+$/CD34$^+$ cells were also detected in vessel endothelium. Additionally, we show that sWAT contains significantly more DLK1$^+$ cells than visceral (v)WAT. We conclude that sWAT has more DKL1$^+$ cells than vWAT and contains different DLK1/CD34 populations, and only cs-DLK1$^-$/cs-CD34$^+$/CD90$^+$/CD105$^{+\,dim}$/α-SMA$^+$/CD45$^-$/CD31$^-$ cells in the adventitial vascular stroma exhibit proliferative and adipogenic capacity.

1. Introduction

White adipose tissue (WAT) is a major energy storage depot and endocrine gland (Gesta et al., 2007). Its physiological functions are mainly fulfilled by adipocytes arising out of adipose-derived stromal/progenitor cells (ASCs) (Yamamoto et al., 2007; Rodeheffer et al., 2008; Tang et al., 2008), which constitute a large pool of adipocyte precursors crucial for adipose tissue homeostasis and expansion (Hausman et al., 2001; Berry et al., 2013). Former studies implicating a predetermined number of fat cells at birth were refuted. In fact, in adult humans ~10% of all fat cells turnover each year (Spalding et al., 2008) and estimates indicate that young adult mice generate ~15% of adipocytes each month (Rigamonti et al., 2011). Different WAT depots, at subcutaneous (sWAT) and visceral (vWAT) sites, comprise distinct adipocyte populations, which are generated by several processes during and after development. These fat cell populations could arise from multiple germ layers and different progenitor cell types (Majka et al., 2011). In fact, it was shown that ASCs reside at the vascular interface and resemble mural cells that can proliferate and differentiate into adipocytes (Tang et al., 2008). Moreover, although controversially discussed, studies suggest that endothelial cells and hematopoietic stem cells might give rise to a subset of adipocytes by changing fate into mural cells and then converting to progenitors (Crossno et al., 2006; Gupta et al., 2012; Medici et al., 2010; Sera et al., 2009; Tran et al., 2012).

Abbreviations: DLK1(PREF1), delta-like protein 1/preadipocyte factor 1; MSC, mesenchymal stem cell; SVF, stromal vascular fraction; WAT, white adipose tissue; CD117, mast/stem cell growth factor receptor (SCFR) also known as c-Kit; CD105, endoglin; CD90, Thy-1; CD45, PTPRC; CD34, cluster of differentiation 34; CD31, cluster of differentiation 31 also known as platelet endothelial cell adhesion molecule (PECAM-1); α-SMA, α-smooth muscle actin; BMI, body mass index; TEM, transmission electron microscopy.

* Corresponding author at: Institute for Biomedical Aging Research, University of Innsbruck, Rennweg 10, 6020 Innsbruck, Austria.
E-mail address: werner.zwerschke@uibk.ac.at (W. Zwerschke).

[1] M.E.Z and A.E contributed equally.

http://dx.doi.org/10.1016/j.scr.2015.08.004

Studies using freshly isolated cells from the stromal vascular fraction (SVF) of sWAT characterized ASCs by their plastic adherence in culture, colony-forming capacity and the expression pattern of distinct cell surface markers (Mitchell et al., 2006). These cells can promote angiogenesis (Rehman et al., 2004) and can be used as autologous ASCs for regenerative therapy (Bourin et al., 2013). It has however not been shown by rigorous assays that ASCs are capable of differentiating outside of their lineage into functional phenotypes without extensive molecular engineering, or extreme chemical treatment (like with BMP, which will temporarily turn any fibroblastic cell into an osteoblast-like cell). The ASC population is broadly defined by the International Society for Cellular Therapy as a subset of cells positive for the mesenchymal stem/stromal cell (MSC) markers CD90 and CD105 (Mitchell et al., 2006), and the stem/progenitor cell marker CD34 (Sengenès et al., 2005; Sidney et al., 2014), lacking the expression of the endothelial marker CD31, summarized as $CD34^+/CD90^+/CD105^+/CD31^-$ (Bourin et al., 2013). There is, however, no consensus about the exact pattern of markers being expressed by ASCs. No definitive singular marker to precisely define ASCs in human adipose tissues is known and although certain MSC markers are recommended, none are specific for a stem cell of any type. They are expressed on virtually all fibroblastic cells that are not stem cells based on rigorous assays (Bianco et al., 2008). According to the current model, in human adipose tissue, vessel-associated stromal cells are organized in two layers, perivascular in an inner ring and more peripheral in an outer ring (Zimmerlin et al., 2013). ASCs reside in the outer adventitial layer encircling small vessels; the spatial organization of ASCs in the vascular stroma of adipose tissues is however not precisely understood.

Delta-like protein 1/preadipocyte factor 1 DLK1(PREF1) is an paternally expressed imprinted gene that encodes a transmembrane protein containing six epidermal growth factor-like repeats at the extracellular domain, a juxtamembrane region with a TNF-alpha-converting enzyme (TACE)-mediated cleavage site, a transmembrane domain and a short intracellular region (Smas and Sul, 1993; Laborda et al., 1993). Upon cleavage by the TACE protease, it can be released as a soluble protein (Sul, 2009). DLK1 is a crucial regulator of ASC homeostasis in WAT. Evidence was presented that DLK1 negatively regulates proliferation (Mortensen et al., 2012) and terminal differentiation of adipocyte progenitors into adipocytes and other mesenchymal lineages (Wang and Sul, 2009). Studies on mouse models confirm these inhibitory effects of DLK1. Animals with increased levels of DLK1 show less total body weight and a decrease in fat mass, whereas DLK1-null mice increases fat mass (Villena et al., 2008; Abdallah et al., 2007; Lee et al., 2003; Mortensen et al., 2012). DLK1 is implicated in stem/progenitor cell biology during embryonic development and in several organs of adults (Floridon et al., 2000; Sul, 2009; Abdallah and Kassem, 2012; Andersen

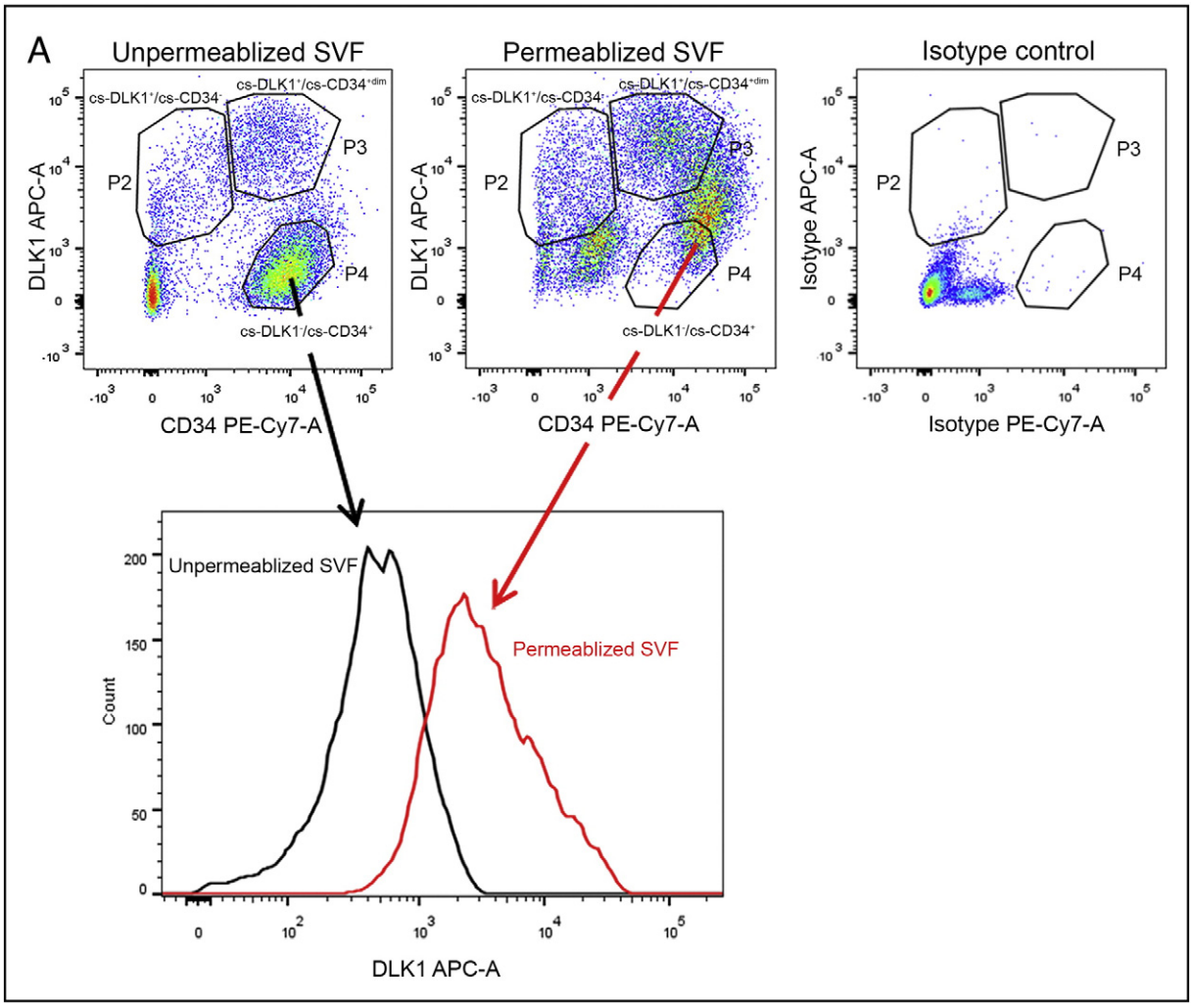


Fig. 1. Detection of $DLK1^+/CD34^+$ cells in the SVF of human sWAT. A. (Upper left panel) Native (unpermeabilized) cells in the SVF were analyzed for cell surface tethered DLK1 and CD34 protein (staining with anti-DLK1 and anti-CD34 antibody without permeabilization and fixation) by FACS. (Upper middle panel) Cells in the SVF were analyzed for intracellular DLK1 and CD34 protein (staining with anti-DLK1 and anti-CD34 antibody after fixation and permeabilization) by FACS. (Upper right panel) Staining of cells in the SVF with isotype control antibodies. (Lower panel) Increasing staining intensity for DLK1 after permeabilization and fixation of SVF cells is shown. B. The cs-$DLK1^+/cs\text{-}CD34^-$ (P2), cs-$DLK1^+/cs\text{-}CD34^{+dim}$ (P3) and cs-$DLK1^-/cs\text{-}CD34^+$ (P4) SVF cell populations (upper panel) were fixed, permeabilized and further analyzed by FACS for the expression of the marker proteins CD90, CD105, α-SMA, CD31 and CD45 (lower panels).

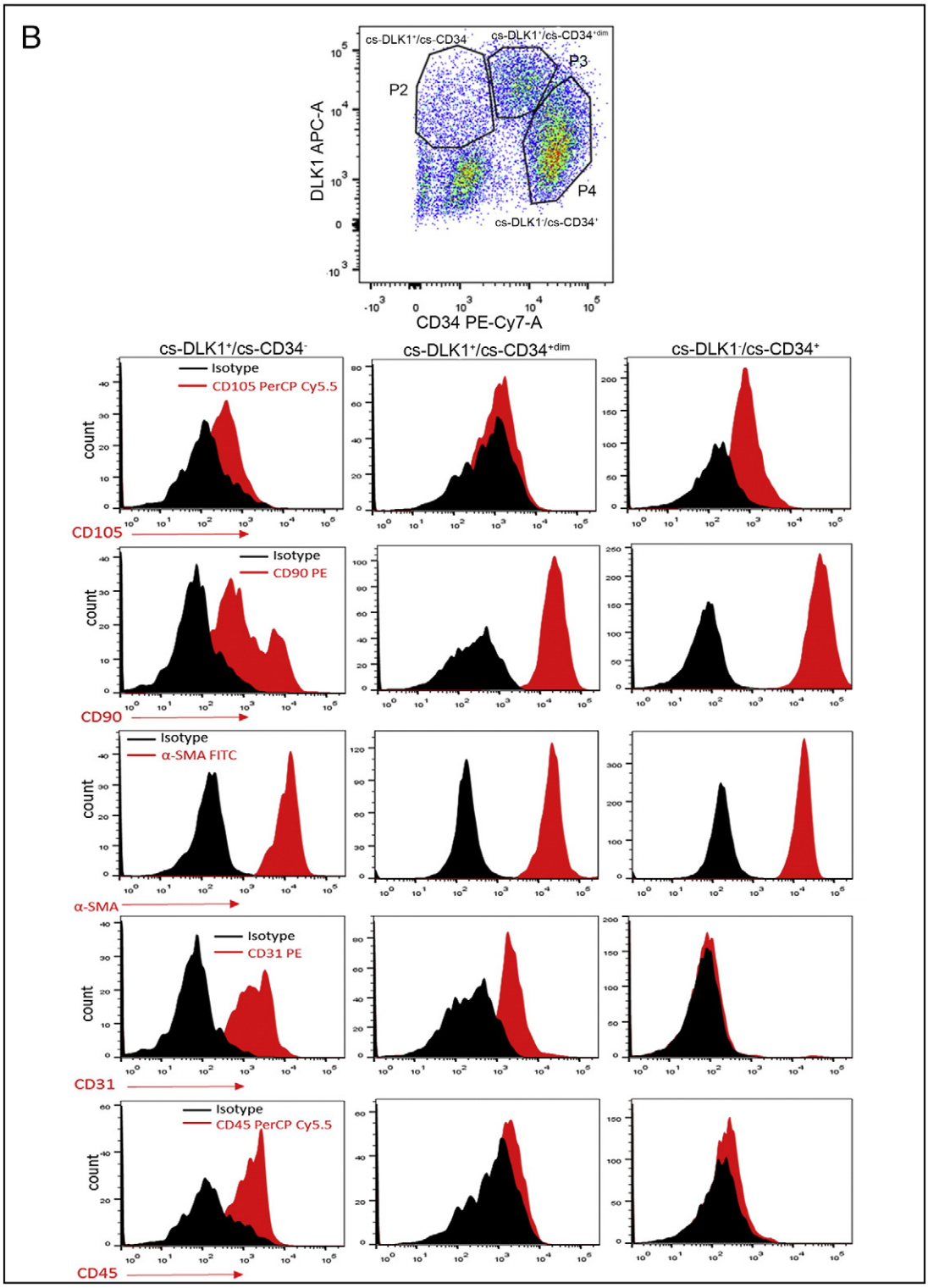


Fig. 1 (*continued*).

et al., 2009b, 2013). In adult mouse fat tissues, adipocyte progenitors express DLK1 and its main role is most likely to regulate clonal expansion and terminal adipogenesis (Sul, 2009; Traustadottir et al., 2013). There is also evidence for a role of DLK1 in vascular endothelium of adipose tissue (Andersen et al., 2009a). We recently showed that DLK1$^+$/CD105$^+$/CD90$^+$/CD34$^+$/CD31$^-$ ASCs constitute a major population in early passage isolates from the SVF of human sWAT and that DLK1 regulates adipogenesis in these cells (Mitterberger et al., 2012). The aim of the present communication was to further characterize DLK1$^+$ ASCs in human WAT.

2. Materials and methods

2.1. Donors

Human adipose tissue samples were either removed from fresh cadavers (n = 18) or taken from patients undergoing routine abdominoplasty (n = 7) at the Institute for Plastic and Reconstructive Surgery (Medical University Innsbruck). The patients gave their informed written consent and had been approved by the ethical

committee of Innsbruck Medical University, Austria, according to the Declaration of Helsinki. Cadavers had been donated to the Department of Anatomy. Due to immediate anonymization of the dead bodies no certificate of non-objection was needed.

2.2. Sample collection

From the Department of Anatomy 15 female and three male cadavers with a post-mortem interval from 4 to 24 h were included in this study. The mean donor age was 85 years at the time of death (range: 63–101 years), showing no apparent disorders in lipid metabolism. Two patients were obese (BMI >30.0), two overweight (BMI 25.0–30.0) and 14 had normal weight (BMI 18.5–25.0). Seven fresh tissue samples from the plastic surgery were obtained from five non-obese, healthy female donors and two male with a mean age of 42 years (range: 28–51 years).

2.3. Cell culture and transfection

ASCs were isolated and cultivated as described (Mitterberger et al., 2014b). Briefly, after surgery adipose tissue biopsies were transferred into sterile serum-free ASC medium (DMEM/F-12 medium (1:1) with HEPES and L-glutamine (Gibco, Vienna, Austria), supplemented with 33 μM biotin, 17 μM pantothenate and 12.5 μg/ml gentamicin and kept at 4 °C for 1–3 h before sterile processing). Fibrous material and blood vessels were dissected. The tissue was cut and collagenase digested. The cells of the stromal-vascular fraction (SVF) were enriched by centrifugation and filtration steps and erythrocyte lysis. The pelletized SVF was suspended in ASC medium supplemented with 10% FBS (Gibco, Vienna, Austria) and filtered again. Aliquots of cells were inoculated at a density of 70,000/cm^2 into 6-well plates. After 16 h of cell attachment, cells were washed with ASC medium and maintained for 6 days in ASC medium without FBS. The remaining cell population was referred to as adipose-derived stromal/progenitor cells (ASCs). For cultivation ASCs were seeded in a density of 5000 cells/cm^2 in ASC medium plus 10% FBS and maintained at 37 °C with 5% CO^2. 16 h later the medium was replaced by PM4 medium (ASC medium containing 2.5% FBS, 10 ng/ml EGF, 1 ng/ml bFGF, 500 ng/ml insulin). ASC were passaged at a ratio of 1:2, medium was changed every third day and the cells were grown to 70% confluence before splitting. HeLa cells (Kaiser et al., 2015) were cultured and transiently transfected with pLenti-DLK1 (Mitterberger et al., 2012) and empty vector as described (Mitterberger et al., 2014a).

2.4. FACS analysis

20 × 10^6 SVF cells were stained with anti-CD34 and anti-DLK1(PREF-1) antibodies and sorted by FACSArIa (BD, Bioscience). Purity of the sorted fractions was analyzed by FACS before functional studies. For surface stainings, cells were stained without fixation while cells were fixed and permeabilized for intracellular stainings.

2.5. Proliferation assay

1 × 10^5 sorted cells from different subsets were seeded in 12-well plates and proliferation of cells was monitored by counting and photographing the cells.

2.6. Colony formation assay

The colony formation assay was done as described (Pochampally, 2008). Briefly, 500 cells were seeded in six well plates as described above. After 10 days of culture colonies derived from single cells were fixed, stained with crystal violet and counted.

2.7. Adipogenic differentiation assay

Sorted fractions were seeded in a 12-well plate at a concentration of 35,000/cm^2 and adipogenesis induced as described (Mitterberger et al., 2012). Adipogenesis was evaluated by analysis of the mRNA expression levels of PPARγ2, FABP4 and Perilipin, and staining the cells with Oil-red-O at day 18 of adipogenesis.

2.8. Cell clots

See supplemental information.

2.9. Sample processing and conventional histology

See supplemental information.

2.10. Antibodies

See supplemental information.

2.11. Immunohistochemistry

See supplemental information.

2.12. Image analysis of immunohistochemistry

See supplemental information.

2.13. Immunofluorescence experiments on paraffin sections

See supplemental information.

Fig. 2. Functional characterization of cs-DLK1$^-$/cs-CD34$^+$, cs-DLK1$^+$/cs-CD34$^-$ and cs-DLK1$^+$/cs-CD34$^{+\,dim}$ cells. A. Protein lysates were prepared from sWAT obtained from the lower abdomen of three different donors, protein concentration determined by BCA protein assay and equal amounts of protein separated by SDS PAGE and analyzed by western blotting using anti-DLK1 antibodies. HeLa cervical cancer cells transfected with empty vector (Mock) and a DLK1 overexpression vector (DLK1 OV) served as controls. B. Subsets of unpermeabilized SVF cells based on surface expression of CD34 and DLK1 were sorted by FACS and purity of the sorted fraction was analyzed. Three populations were isolated, cs-DLK1$^-$/cs-CD34$^+$, cs-DLK1$^+$/cs-CD34$^-$ and cs-DLK1$^+$/cs-CD34$^{+\,dim}$ cells. C. Protein lysates from cs-DLK1$^+$/cs-CD34$^-$ and cs-DLK1$^+$/cs-CD34$^{+\,dim}$ cells were prepared in SDS sample buffer containing DTT and β-mercaptoethanol, protein concentration determined by BCA protein assay. Equal amounts of protein were separated by SDS PAGE and analyzed by western blotting using anti-DLK1 antibodies. β-Actin served as input control. D. Analysis of DLK1 (left panel) and CD34 mRNA expression (right panel) in cs-DLK1$^-$/cs-CD34$^+$, cs-DLK1$^+$/cs-CD34$^-$ and cs-DLK1$^+$/cs-CD34$^{+\,dim}$ cells. mRNA expression was measured relative to actin using q-RT-PCR analysis (n = 4). E. Proliferative capacity of the different DLK1/CD34 cell types isolated from the SVF. 1 × 10^5 sorted cells from different subsets were seeded in 12-well plates and proliferation of cells was monitored by cell counting for 7 days. Representative Photographs 6 days after seeding are shown. F. Colony formation assay. (Left panel) 500 cells of each cell type were seeded, cultured for 10 days, fixed and stained with crystal violet. (Right panel) The number of colonies formed from single cells after 10 days is indicated. G. Adipogenic differentiation capacity of the different DLK1/CD34 cell types isolated from the SVF. (Upper panel) Indicated cell types were subjected to the adipogenic differentiation protocol and differentiated cells were detected by Oil-red-O staining and light microscopy. 400 cells were counted, 39 +/− 3% of cs-DLK1$^-$/CD34$^+$ cells differentiated into adipocytes. (Lower panel) Adipogenic differentiation in the cs-DLK1$^-$/CD34$^+$ cells was also monitored by q-RT PCR by analyzing the expression of the adipogenic key regulator PPARγ2 and of the adipocyte differentiation markers FABP4 and Perilipin at day 21 post induction. Moreover, Perilipin protein levels were monitored by western blot analysis. For the mRNA expression analysis, the expression level at day 0 was defined to be 1. Error bars = S.E.M., n = 4.

2.14. Image analysis of immunofluorescence

See supplemental information.

2.15. Preembedding immunoperoxidase stainings for light and transmission electron microscopy (TEM)

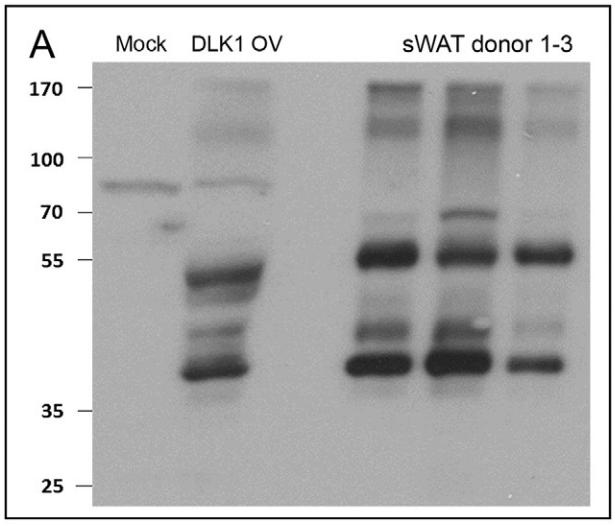
See supplemental information.

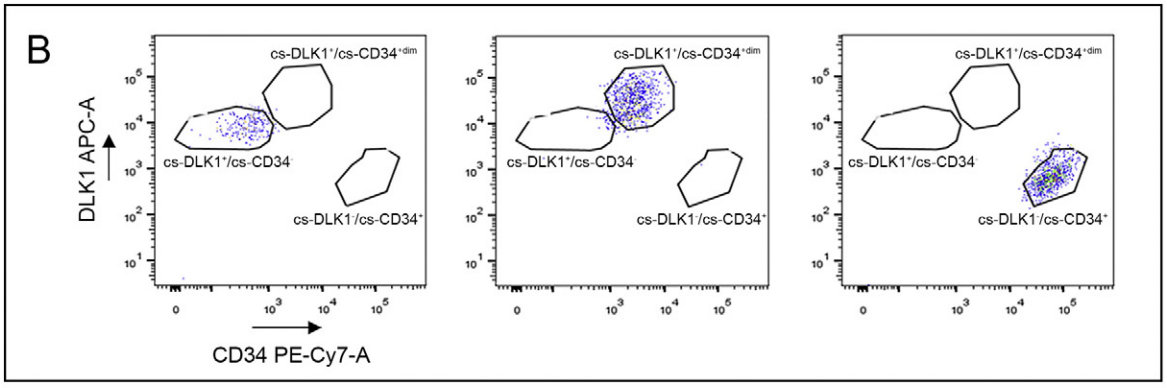
2.16. Image analysis of preembedding immunoperoxidase stainings on TEM level

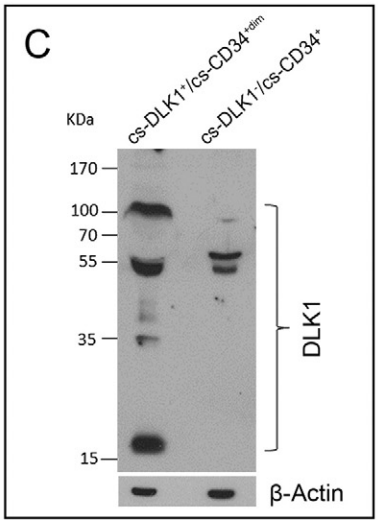
See supplemental information.

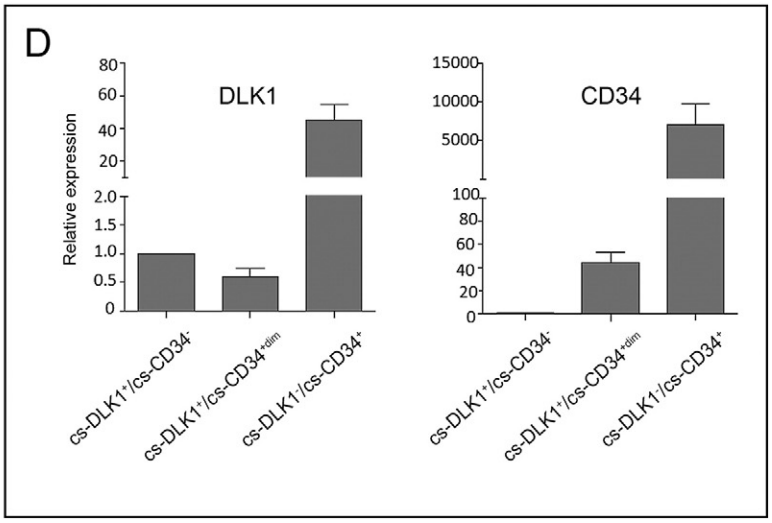
2.17. Western blot analysis

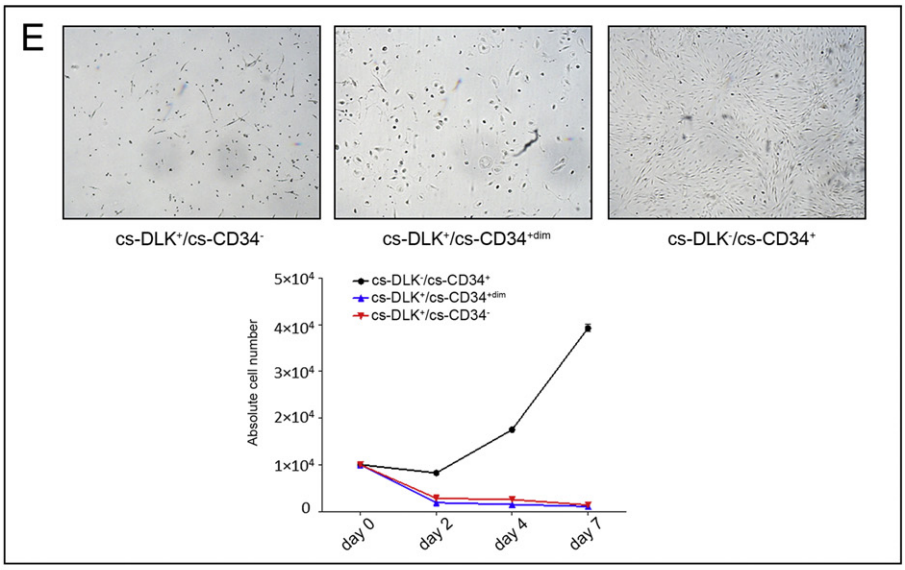
See supplemental information.

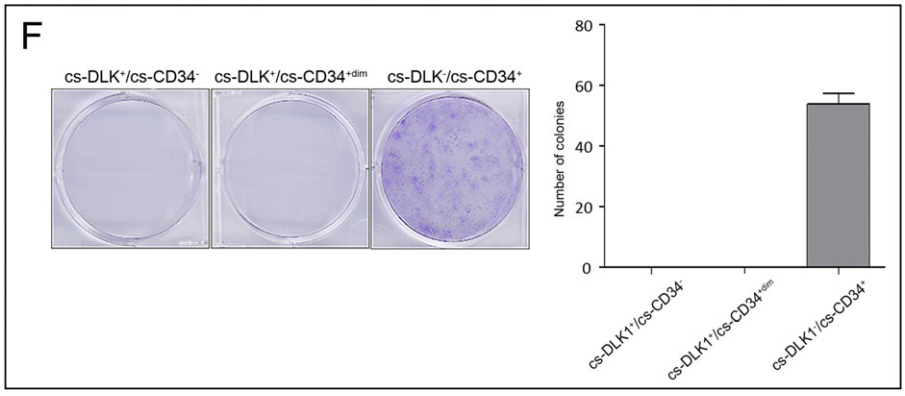












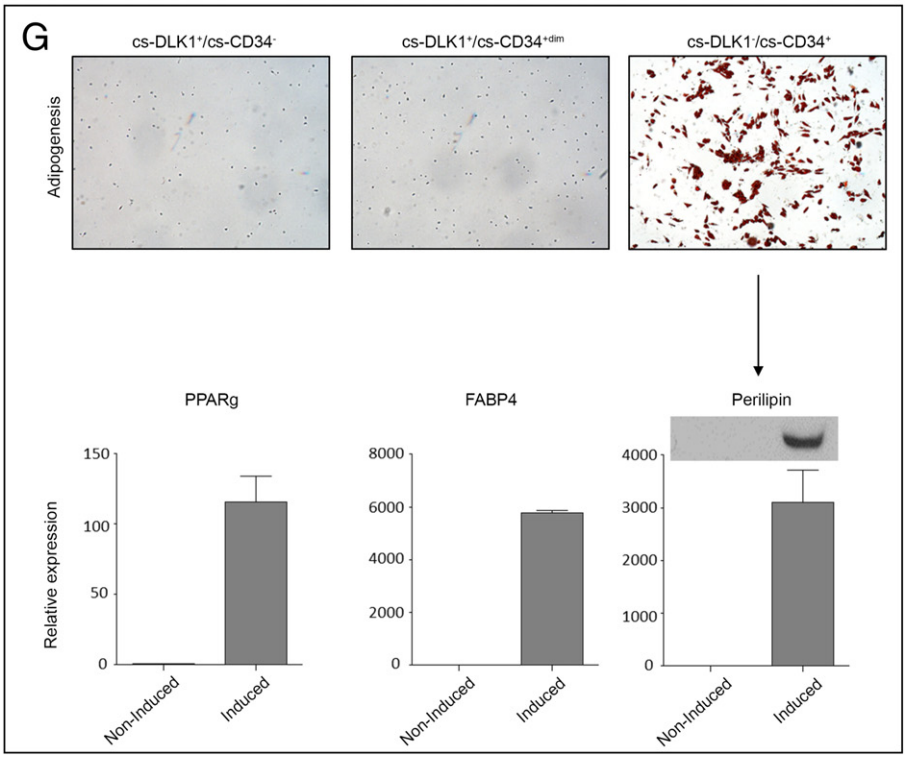


Fig. 2 *(continued).*

2.18. Quantitative real-time PCR analysis

Q-RT PCR analysis was conducted as described (Lechner et al., 2013). See supplemental information for the primer list.

2.19. Comparison of DLK1$^+$ cells in sWAT and vWAT and statistical analysis

See supplemental information.

3. Results

3.1. Characterization of DLK1$^+$/CD34$^+$ cells in the SVF of human sWAT

To better understand the importance of DLK1 for ASCs we sorted native (unpermeabilized) SVF cells from sWAT for cell surface staining (cs) of DLK1 and CD34 (Fig. 1A). This approach identified three different cell populations: 10.1% of all SVF cells were cs-DLK1$^+$/cs-CD34$^-$ (population, P2), 19.8% were cs-DLK1$^+$/cs-CD34^{+dim} (P3) and 43.4% stained cs-DLK1$^-$/cs-CD34$^+$ (P4) (Fig. 1A, upper left panel). Analysis of permeabilized SVF cells revealed strong intracellular staining for DLK1 in P4 cells (Fig. 1A, upper middle panel and lower panel). As expected, the DLK1 staining intensity of P2 and P3 cells also increased, indicating that all three populations contain intracellular DLK1 protein. No intracellular staining for CD34 was detected in P2 (Fig. 1A, upper middle panel). The SVF contains CD90$^+$ (data not shown) and CD105^{+dim} cells (Supplemental Fig. 1). Further FACS analysis of permeabilized cells showed that cs-DLK1$^-$/cs-CD34$^+$ cells (P4) (Fig. 1B, upper panel), expressed the two mesenchymal marker proteins (CD90$^+$ and CD105^{+dim}) (Fig. 1B, lower right panels), albeit CD105 staining intensity in SVF cells was relatively low. The CD105 level increased during in vitro expansion (Supplemental Fig. 1). These cells stained also positive for the pericyte marker α-smooth muscle actin (α-SMA) but expressed neither the hematopoietic lineage marker protein CD45 nor the endothelial cell marker protein CD31 (Fig. 1B, lower right panels). Thus, we nomenclatured these cells in the SVF as cs-DLK1$^-$/cs-CD34$^+$/CD90$^+$/CD105^{+dim}/α-SMA$^+$/CD45$^-$/CD31$^-$ ASCs. The two populations with cell surface-tethered DLK1 protein (Fig. 1B, upper panel), which co-expressed either no CD34 (cs-DLK1$^+$/cs-CD34$^-$) (P2) or low levels of CD34 on the surface (cs-DLK1$^+$/cs-CD34^{+dim}) (P3), stained also positive for α-SMA but differed in the expression of the other marker proteins (Fig. 1B, lower left and middle panels). Cs-DLK1$^+$/cs-CD34$^-$ cells stained positive for CD45, CD90 and dimly positive for CD105 (Fig. 1B, lower left panels). Cs-DLK1$^+$/cs-CD34^{+dim} cells were negative for CD45 and CD105 but positive for CD90 (Fig. 1B, lower middle panels). We conclude, cs-DLK1$^+$ cells express mesenchymal, pericyte and hematopoietic markers and no or low levels of CD34. Interestingly, both cs-DLK1$^+$ cell populations were positive for the endothelial marker CD31 (Fig. 1B, lower left and middle panels), comprising the immune phenotypes cs-DLK1$^+$/cs-CD34$^-$/CD90$^+$/CD105^{+dim}/ α-SMA$^+$/CD45$^+$/CD31$^+$ and cs-DLK1$^+$/cs-CD34^{+dim}/CD90$^+$/CD105$^-$/α-SMA$^+$/CD45$^-$/CD31$^+$. Thus only the large SVF fraction of cs-DLK1$^-$/cs-CD34$^+$/CD90$^+$/CD105^{+dim}/ α-SMA$^+$/CD45$^-$/CD31$^-$ cells possessed a mesenchymal and pericyte-like phenotype. The other two cell populations showed mixed expression of mesenchymal, pericyte, hematopoietic and endothelial markers.

3.2. Cs-DLK1$^-$/cs-CD34$^+$ cells but neither cs-DLK1$^+$/cs-CD34$^-$ nor cs-DLK1$^+$/cs-CD34^{+dim} cells undergo clonal expansion and terminal adipogenic differentiation

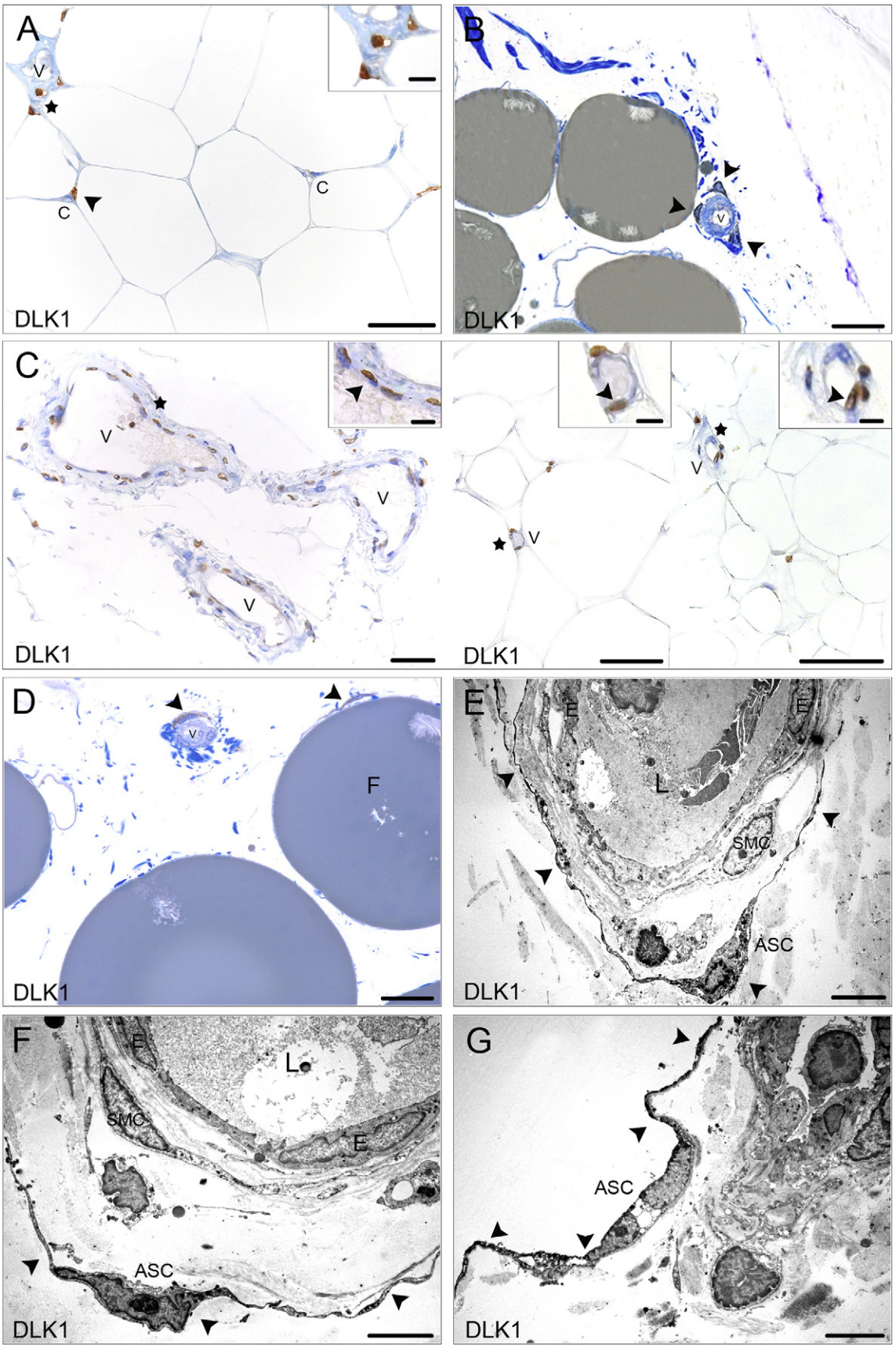
Multiple isoforms of DLK1 are described, due to posttranslational modifications and alternative splicing. The abundance of these isoforms varies depending on tissue and cell type (da Rocha et al., 2009; Ferrón et al., 2011; Lee et al., 1995; Mei et al., 2002; Mortensen et al., 2012; Smas et al., 1994; Sul, 2009). We show that in human sWAT the 55 kDa and a ~40 kDa DLK isoforms are most abundant (Fig. 2A). Next we isolated the cs-DLK1$^-$/cs-CD34$^+$, cs-DLK1$^+$/cs-CD34$^-$ and cs-DLK1$^+$/cs-CD34^{+dim} cell populations by FACS sorting of native cells from the human SVF (Fig. 2B) and compared DLK1 protein isoforms and abundance in cs-DLK1$^-$/cs-CD34$^+$ and cs-DLK1$^+$/cs-CD34^{+dim} cells. DLK1 protein was detected in both cs-DLK1$^+$ as well as in cs-DLK1$^-$ cells (Fig. 2C). The overall DLK1 protein levels in cs-DLK1$^+$ cells were however much higher than in cs-DLK1$^-$ ASCs and we detected different DLK1 isoform pattern in the given cell types. The level of the 55 kDa DLK1 isoform was higher in cs-DLK1$^-$ ASCs than in cs-DLK1$^+$ ASCs, while a 50 kDa isoform was more abundant in cs-DLK1$^+$ cells. Intriguingly, a highly abundant large isoform of the DLK1 protein (~110 kDa) was detected in cs-DLK1$^+$ cells but not in cs-DLK1$^-$ ASCs (Fig. 2C). Since SDS-PAGE/western blot was done in reducing conditions, this band results most likely not from dimer formation by disulfide bridges although the DLK1 protein contains multiple cysteine residues. Moreover, some faint DLK1 protein bands between 35 and 40 kDa and a ~20 kda band could only be detected in cs-DLK1$^+$ cells. Interestingly, we found that the DLK1 mRNA expression levels in the given ASC subtypes did not correlate with DLK1 protein levels, as demonstrated by q-RT PCR analysis (Fig. 2D). This underscores that the DLK1 protein levels in human ASCs are regulated at the post transcriptional level. In fact, DLK1 mRNA expression was approximately 40 times higher in cs-DLK1$^-$/cs-CD34$^+$ cells than in cs-DLK1$^+$/cs-CD34$^-$ or cs-DLK1$^+$/cs-CD34^{+dim} cells, which contain high level of cell surface bound DLK1 protein. This suggests that the turnover of DLK1 protein in cs-DLK1$^-$/cs-CD34$^+$ ASCs is higher. As expected CD34 mRNA expression was strongly up-regulated in cs-DLK1$^-$/cs-CD34$^+$ cells relative to cs-DLK1$^+$/cs-CD34$^-$ ASCs. These data corroborate that the given cell types express DLK1 and CD34.

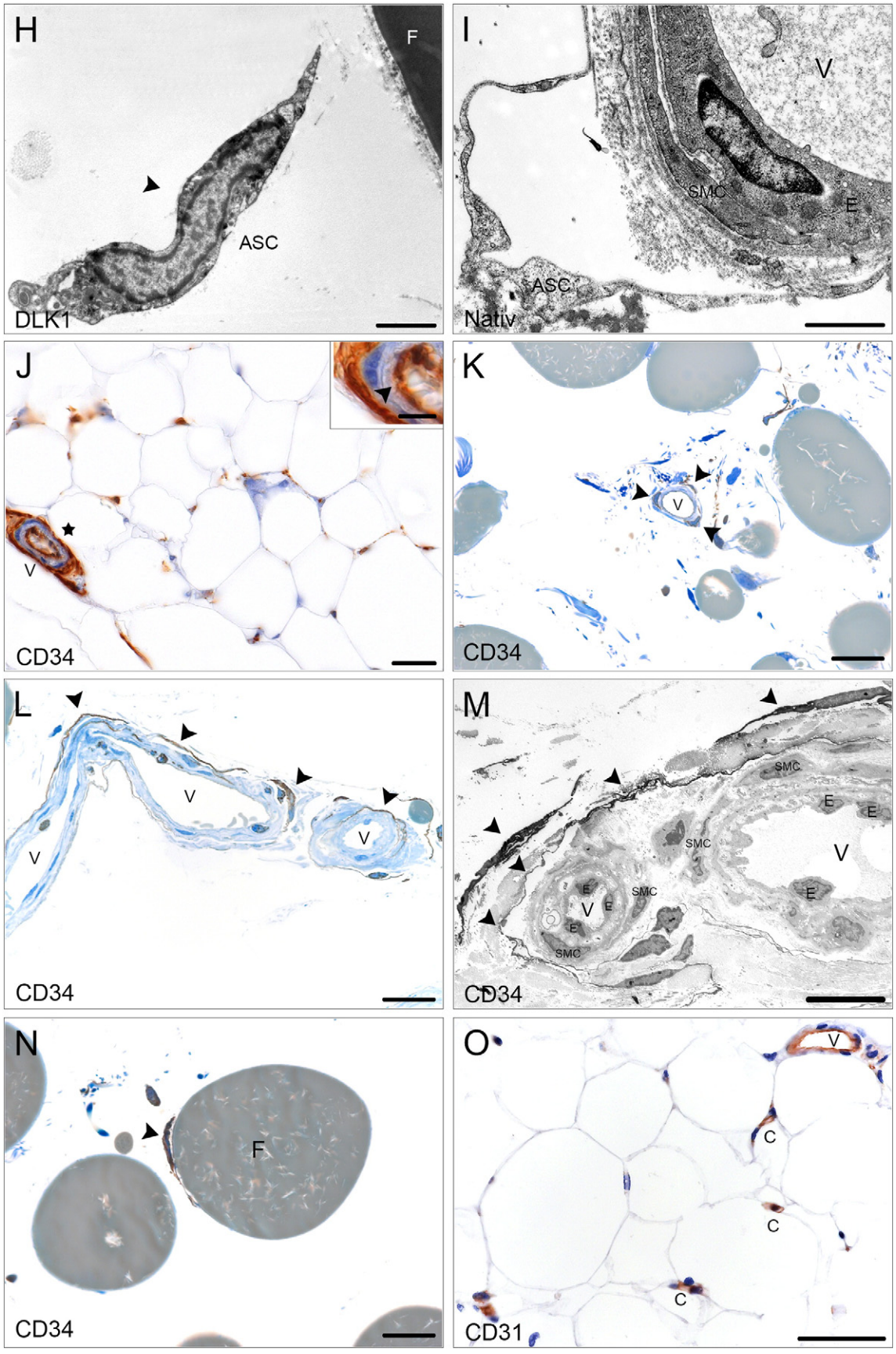
We analyzed the proliferative and adipogenic capacity of the different DLK1/CD34 cell populations isolated by sorting of native cells from the human SVF (Fig. 2B). Interestingly, we found that only cs-DLK1$^-$/cs-CD34$^+$ (P4) cells showed strong proliferation (Fig. 2E and F) and high terminal adipogenic differentiation capacity (Fig. 2G), while cs-DLK1$^+$/cs-CD34$^-$ (P2) and cs-DLK1$^+$/cs-CD34^{+dim} (P3) cells could neither proliferate (Fig. 2E and F) nor differentiate into adipocytes (Fig. 2G). Together our data suggest that only SVF cells without cell surface-tethered DLK1, but with cell surface-bound CD34, can undergo clonal expansion and terminal adipogenesis, while cell surface-tethered DLK1 has inhibitory effects. Similarly, a previous study by Mortensen et al. (2012) showed that only non-cleavable cell surface-tethered DLK1 isoforms inhibit 3T3-L1 preadipocyte proliferation. While this supports the model that adipose tissue expansion occurs through proliferation and terminal adipogenic differentiation of preadipocytes, future studies are necessary to better understand the nature and role of the different DLK1 isoforms in human ASCs.

3.3. Localization of DLK1$^+$/CD34$^+$ cells in intact human sWAT

We characterized the spatial organization of DLK1$^+$ ASCs in intact human sWAT by immunohistochemistry (IHC) using a mouse monoclonal anti-DLK1 antibody (Supplemental Fig. 2A and B). This antibody detected the majority of DLK1$^+$ cells in the outer adventitial ring associated with vascular stroma and around capillaries (Fig. 3A and B). A significant number of cells in the endothelial layers lining small vessels stained also positive for DLK1 (Fig. 3C, left and right panel). Moreover, DLK1$^+$ cells were less frequently located in close proximity to fat cells (Fig. 3D). The morphology of DLK1$^+$ cells was studied using native as well as pre-embedding transmission electron microscopy (TEM). The ultra-structural analyses demonstrated that DLK1$^+$ cells were characterized by a spindle-shape and extremely long cytoplasmic processes (Fig. 3E–H). According to their location and morphology, these cells could also be natively identified (Fig. 3I). CD34$^+$ cells were abundantly found in the outer adventitial ring associated with vascular stroma and around capillaries (Fig. 3J–M). In addition CD34$^+$ cells were detected in many but not all endothelial layers lining small vessels (Fig. 3J). Some

$CD34^+$ cells were located in close proximity to fat cells (Fig. 3N). Single staining using indirect immunofluorescence and a confocal laser scanning system detected also $DLK1^+$ (Fig. 4A–C, arrow heads) and $CD34^+$ (Fig. 4D–F) cells in the vascular stroma and in the endothelial layer of small vessels of sWAT, corroborating our IHC results. Additional CD31 staining was conducted to visualize the endothelium (Figs. 3O–Q





and 4G–I). Co-stainings mainly detected cells positive for both CD34 and DLK1 in the outer adventitial ring associated with vascular stroma and around capillaries (Fig. 3R, black asterisks, black arrow heads in inserts). $CD34^{+}/DLK1^{+}$ double-positive cells were also detected in endothelial layers of small vessels (Fig. 3R, upper panels, white asterisks, black arrow heads in inserts). These data complement the

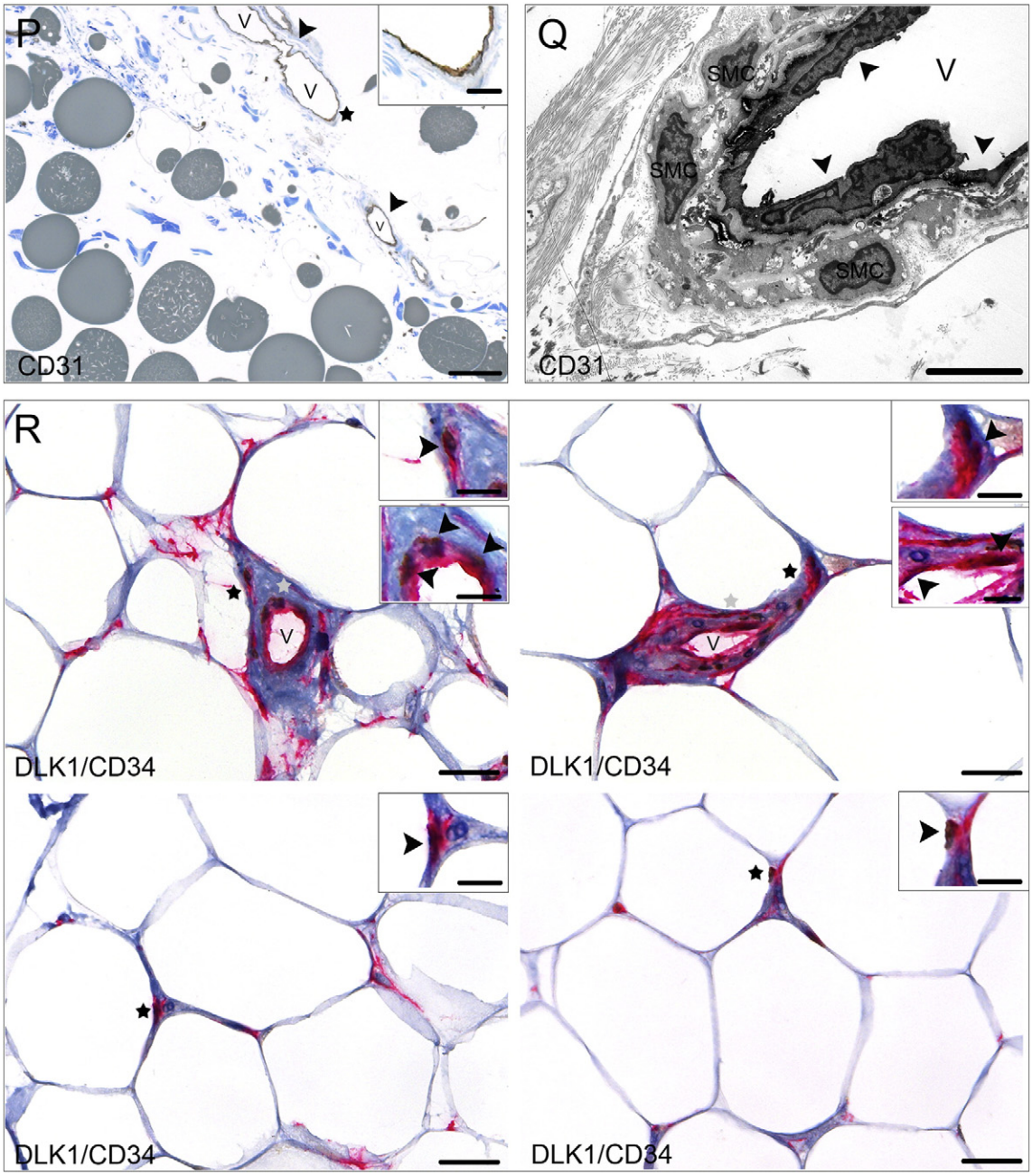


Fig. 3. Detection of DLK1$^+$ and CD34$^+$ cells in intact human sWAT. A. IHC staining of sWAT paraffin sections using antibodies against DLK1. DLK1$^+$ cells surrounding vessels and next to capillaries are shown. Arrow head marks the cell next to a capillary. Inset shows DLK1$^+$ cells in higher magnification. Asterisk indicates the localization of the inserts. V, vessel, C, capillary. Bar: 50 μm. Inset: 10 μm. B. Light microscope micrograph of a semi-thin sWAT section. Location of DLK1$^+$ cells around small vessels in sWAT (arrow heads). V, vessel. Bar: 25 μm. C. IHC stainings of cells in the inner endothelial ring of blood vessels in sWAT using antibodies against DLK1. V, vessel. Bar: right panel 50 μm and left panel 20 μm. Inset: 5 μm. D. Light microscope micrograph of a semi-thin sWAT section. Location of DLK1$^+$ cells around small vessels and more peripherally between fat cells are shown (arrow heads). V, vessel, F, fat cell. Bar: 25 μm. E–I. Morphology of DLK1$^+$ ASCs in fresh sWAT. Pre-embedding electron microscopy on ultra-thin sections shows DLK1$^+$ cells (arrow heads), located in the adventitial region of the vasculature (E–G) or more peripherally (H). Note the long cytoplasmatic processes of these cells. V, vessel, E, endothelial cell, ASC, adipose-derived stromal/progenitor cell, SMC, smooth muscle cell, F, fat cell. (I) Native electron microcopy. L, lumen, E, endothelial cell, SMC, smooth muscle cell, ASC, adipose-derived stromal/progenitor cell. Bar: E–G (5 μm), H (2 μm), I (1 μm). J. IHC staining of sWAT paraffin sections using antibodies against CD34. CD34$^+$ cells surrounding vessels and next to capillaries are shown. Inset shows CD34$^+$ endothelial cells (arrow head) in higher magnification. Asterisk indicates the localization of the insert. V, vessel. Bar: 50 μm. Inset: 10 μm. K. Light microscope micrograph of a semi-thin sWAT section. Location of CD34$^+$ cells around small vessels in sWAT is shown (arrow heads). V, vessel. Bar: 25 μm. L. Light microscope micrograph of a semi-thin sWAT section. CD34+ cells (arrow heads) are present around vessels. V, vessels. Bar: 25 μm. M. Pre-embedding electron microscopy showing the location and morphology of CD34$^+$ cells (arrow heads) in the outer adventitial ring of small blood vessels. E, endothelial cells, V, vessel, SMC, smooth muscle cell. Bar: 10 μm. N. Light microscope micrograph of a semi-thin sWAT section. A CD34$^+$ cell (arrow head) present more peripherally between fat cells is shown. F, fat cell. Bar: 25 μm. O. IHC staining of endothelial cells marking the inner ring of small blood vessels and capillaries in human sWAT sections by an anti-CD31 antibody. V, vessel, C, capillary. Bar: 50 μm. P. Vessels (arrow heads) are shown in a semi-thin section of WAT. Asterisk indicates the localization of the insert. Note that endothelium cells are CD31 positive. V, vessel. Bar: 50 μm. Insert: 5 μm. Q. Pre-embedding electron microscopy marking the endothelium (arrow heads) of small blood vessels. V, vessel, SMC, smooth muscle cell. Bar: 5 μm. R. Double-staining IHCs for DLK1 (brown) and CD34 (red). Inserts show stromal cells co-containing both DLK1 and CD34 protein (arrow heads). Asterisks indicate the localization of the inserts. DLK1$^+$/CD34$^+$ double positive cells were also detected in the endothelial layers lining the small vessels (V), indicated by arrows. Bar: 25 μm. Insert: 10 μm.

FACS analysis, which detected significant numbers of DLK1$^+$/CD34$^+$/CD31$^-$ and DLK1$^+$/CD34$^{+\,dim}$/CD31$^+$ cells in the SVF (Fig. 1B). Although the IHC analysis is not quantitative the histological data suggest that DLK1$^+$ cells copy the same location and morphology as CD34$^+$ cells in the outer adventitial ring, around capillaries and in vascular endothelia of intact sWAT.

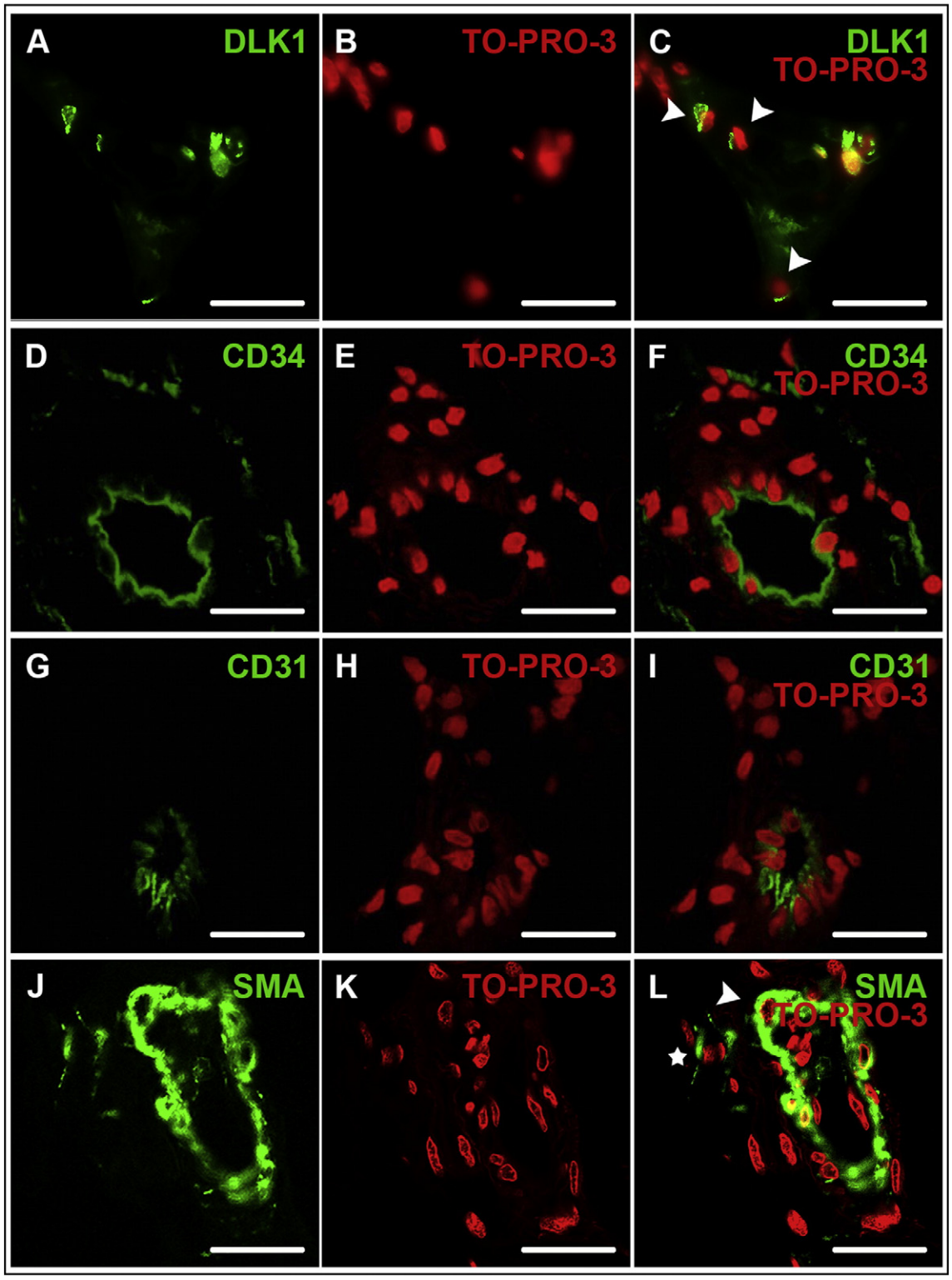
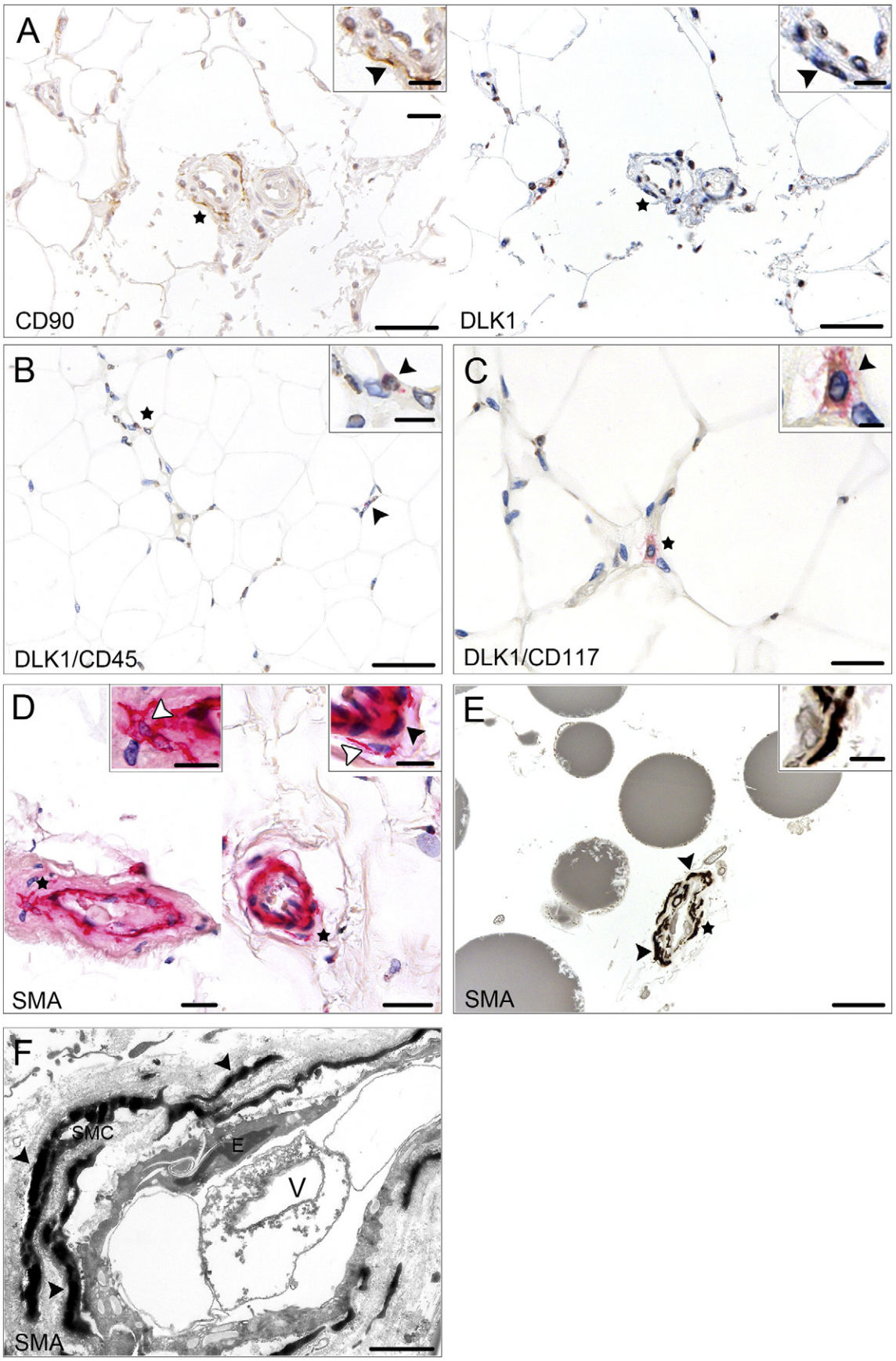


Fig. 4. Detection of DLK1, CD34, CD31 and α-SMA positive cells in human sWAT using indirect immunofluorescence and a confocal laser scanning system. Detection of DLK1 (A–C, arrow heads), CD34 (D–F), CD31 (G–I), and α-SMA (J–L) in sWAT. Positive cells (green) are shown in relation to their location to vasculature in sWAT. TO-PRO-3 (nuclei) (red). Bar: A–L (25 μm).

We investigated whether DLK1$^+$ cells in intact sWAT stained positive for mesenchymal and hematopoietic lineage marker proteins. To make precise comparison of the same cells stained for CD90 and DLK1 proteins, two consecutive "mirror" sections were stained by IHC, which allows separate staining of the same section surface by two different antibodies (Nakamura et al, 1999). This procedure demonstrated a high abundance of DLK1$^+$/CD90$^+$ cells in stroma surrounding small vessels and around capillaries (Fig. 5A). In fact, the majority of DLK1$^+$ cells were co-stained with the mesenchymal marker CD90. Co-staining of DLK1 with the hematopoietic lineage marker CD45 (Fig. 5B) and the hematopoietic stem cell marker CD117(c-Kit) (Fig. 5C) led to the detection of DLK1$^+$/CD45$^+$ and DLK1$^+$/CD117$^+$ double-positive cells in

intact sWAT, which were however much less abundant than DLK1$^+$/CD90$^+$ cells and only sporadically distributed throughout the adipose tissue.

To analyze whether DLK1$^+$ cells show pericyte features we employed α-SMA antibodies. α-SMA$^+$ cells were mainly located perivascular next to the endothelium and to a lesser extent more



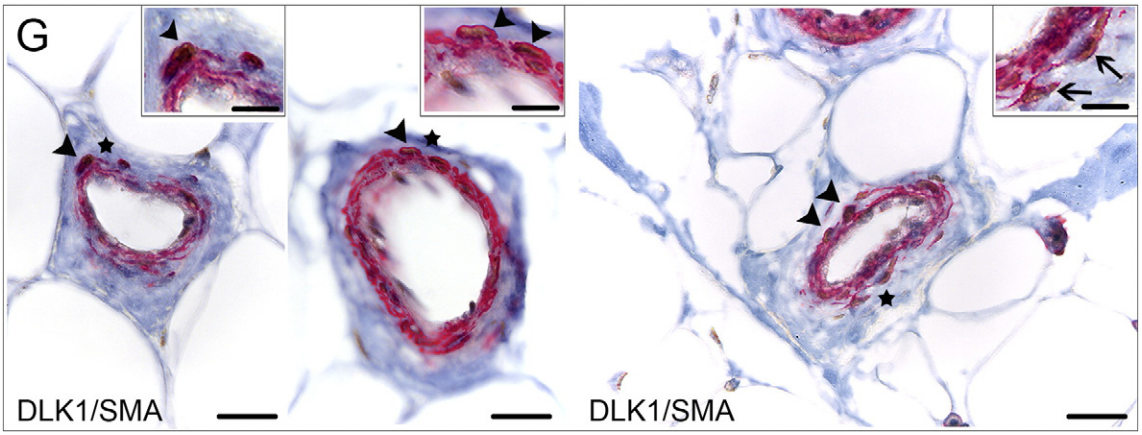


Fig. 5. Co-staining of DLK1$^+$ cells with CD90, CD45, CD117, and α-SMA in intact human sWAT. A: Consecutive "mirror" sections of human sWAT using antibodies against CD90 (left panel and arrow head) and DLK1 (right panel and arrow head) demonstrates localization of both proteins in the same cell. Asterisks indicate the localization of the inserts. B and C: Double-staining IHCs for DLK1 (brown)/CD45 (red) and DLK1 (brown)/CD117 (red) showing cells double-positive for both markers (arrow head). Asterisks indicate the localization of the inserts. D: IHC stainings demonstrate α-SMA positive cells around blood vessels. Inserts show α-SMA positive cells in the adventitial ring (white arrow heads) and pericytes (black arrow head). Asterisk indicates the localization of the insert. E: Pericytes/smooth muscle cells (arrow heads) are shown in a semi-thin section of WAT. Inset showing the location of α-SMA positive cells next to an endothelial cell. Asterisk indicates the localization of the insert. Section was not counterstained with toluidine blue. F: Pre-embedding electron microscopy marking α-SMA positive cells (arrow heads) and their location around small blood vessels/capillaries. V, vessel, E, endothelial cell, SMC, smooth muscle cell. G: Double-staining IHC showing DLK1$^+$/α-SMA$^+$ pericytes (arrow heads) and DLK1$^+$/α-SMA$^+$ cells located more peripherally in the adventitial ring (arrows). Asterisks indicate the localization of the inserts. Bar: A and B (50 μm), C, D, E and G (25 μm), F (3 μm), Inset: D (15 μm), A, B and G (10 μm), and C and E (5 μm).

peripheral in the adventitia (Figs. 4J–L, 5D). The employment of pre-embedding techniques corroborated these findings, cells positive for α-SMA were mainly located perivascular (Fig. 5E and F). Co-staining with anti-α-SMA and anti-DLK1 antibodies detected DLK1$^+$/α-SMA$^+$ cells perivascular in the inner ring of vessel-associated stromal cells (Fig. 5G, arrow heads) but also in the outer adventitial ring, relative close to the perivascular ring (Fig. 5G, right panel, arrows). These data suggest a high abundance of DLK1$^+$/α-SMA$^+$ cells in the vessel stroma of intact sWAT and complement our FACS analysis, which indicated that the cs-DLK1$^-$/cs-CD34$^+$/CD31$^-$ cell population in the SVF is α-SMA$^+$ (see below, Fig. 1B). Moreover, the FACS analysis detected cs-DLK1$^+$/cs-CD34$^-$/α-SMA$^+$/CD31$^+$ cells in the SVF, which would be congruent with our IHC detection of DLK1$^+$/α-SMA$^+$ cells in the CD34$^-$ perivascular ring.

3.4. Visceral WAT depots contain less DLK1$^+$ cells than subcutaneous WAT depots

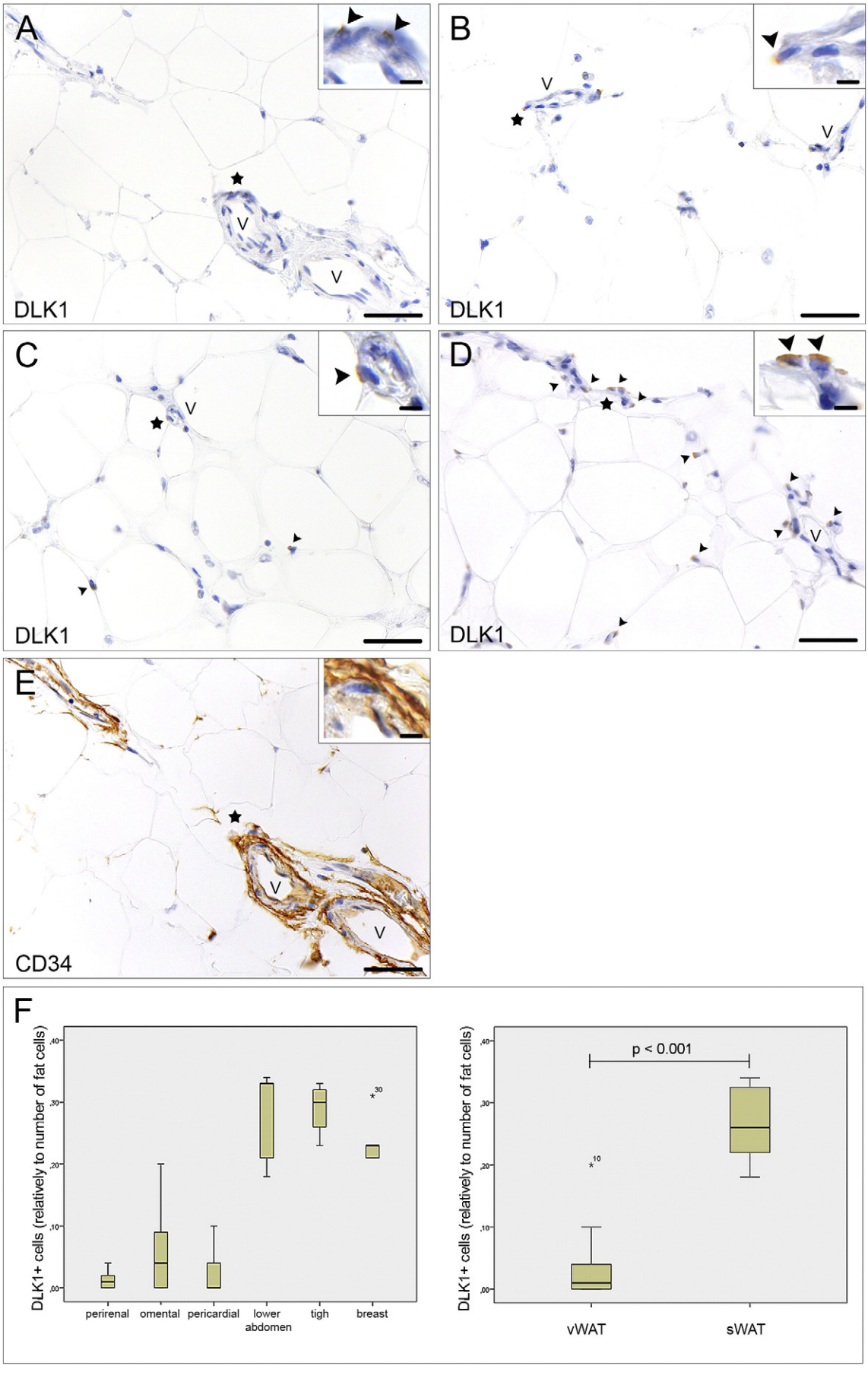
DLK1 expression in human vWAT depots is unknown. To address this question, we stained paraffin sections of different vWAT and sWAT depots derived from 10 donors using anti DLK1 antibodies and compared the number of DLK1$^+$ cells (Fig. 6A–D). We found that the numbers of DLK1$^+$ cells in omental, perirenal and pericardial vWAT depots were significantly lower than in sWAT depots (lower abdomen, thigh and breast) (6 F), although DLK1$^+$ cells could be sporadically detected around capillaries and small vessels in all vWAT depots. Consecutive sections from the omental vWAT depot stained by DLK1 (Fig. 6A) and CD34 (Fig. 6E) demonstrated that only the number of DLK1$^+$ cells was reduced in the visceral fat depot however not the number of CD34$^+$ cells.

4. Discussion

Renewal of adipose tissue from progenitor cells is important for its homeostasis throughout life. DLK1-positive adipose precursors among others are required for adipose tissue expansion in adult mice Hudak et al. (2014). The mechanisms regulating expansion of the adipogenic lineage and terminal adipogenesis are however little understood, especially in human adipose tissues. In the present study, we demonstrated for the first time that the SVF of human sWAT contains three different cell populations defined by DLK1 and CD34. Only cs-DLK1$^-$/cs-CD34$^+$/CD90$^+$/CD105$^{+\,dim}$/α-SMA$^+$/CD45$^-$/CD31$^-$ cells showed high proliferative and adipogenic capacity. Cs-DLK1$^+$/cs-CD34$^-$ and cs-DLK1$^+$/cs-CD34$^{+\,dim}$ cells, which bear cell-surface-tethered DLK1, consistently co-expressed α-SMA and CD31 and had a mixed hematopoietic and mesenchymal phenotype, could neither undergo proliferation nor terminal adipogenesis. While previous studies underscore that DLK1 restricts adipose tissue size by inhibiting clonal expansion and terminal adipogenesis of ASCs (Sul, 2009; Traustadottir et al., 2013), our data suggest that cell surface expression of DLK1 plays a role in the regulation of clonal expansion and terminal adipogenesis of human ASCs. Further work is necessary to elucidate the role of intracellular DLK1.

To histologically characterize DLK1$^+$ cells in the vascular stroma of intact human sWAT we combined DLK1 in situ staining with stainings of different stem/progenitor and lineage marker proteins. According to the current model, human ASCs are positive for the stem/progenitor cell marker CD34$^+$ and mainly localized in the adventitia of small vessels (Sengenès et al., 2007; Zimmerlin et al., 2013). In the present study, we corroborate these data, demonstrate for the first time a high abundance of DLK1$^+$/CD34$^+$ cells in the SVF derived from human sWAT and show localization of those cells in the outer adventitial stromal ring of small vessels and at capillaries. The vast majority of DLK1$^+$ cells localized in this region stained also positive for CD90, indicating the mesenchymal character of the DLK1$^+$/CD34$^+$ cells.

Interestingly, we detected DLK1$^+$/α-SMA$^+$ cells perivascular and in the outer adventitial stromal ring. α-SMA marks pericytes and vascular smooth muscle cells, also referred to as mural cells, which reside mainly perivascular (Cai et al., 2011; Corselli et al., 2012; Lin et al., 2008; Maumus et al., 2011). Increasing evidence suggest that CD34$^+$ ASCs in the vascular stroma, which are required for adipose tissue maintenance, arise from a α-SMA$^+$ mural lineage (Tang et al., 2008; Jiang et al., 2014; Zimmerlin et al., 2013). Our IHC results show that DLK1$^+$/α-SMA$^+$ cells are localized in the CD34$^-$ perivascular inner ring of vessel-associated stroma. This matches with the detection of cs-DLK1$^+$/cs-CD34$^-$/α-SMA$^+$ SVF cells by FACS analysis. IHC analysis detected also DLK1$^+$/α-SMA$^+$ cells at the interphase between perivascular ring and adventitia and in the adventitial vascular stroma, which is in accordance with the detection of cs-DLK1$^-$/cs-CD34$^+$/α-SMA$^+$ SVF cells by FACS analysis. Thus, it is conceivable that such cells emerge during transition from a cs-DLK1$^+$/cs-CD34$^-$/α-SMA$^+$ pericyte lineage to cs-DLK1$^-$/cs-CD34$^+$/α-SMA$^+$ ASCs. In accordance with this hypothesis, we found that cs-DLK1$^-$/cs-CD34$^+$/CD90$^+$/CD105$^{+\,dim}$/α-SMA$^+$/CD45$^-$/CD31$^-$ cells constitute a major cell population in the vascular stroma of sWAT, which exhibits preadipocyte properties.


A
V
V
DLK1
B
V
V
DLK1
C
V
DLK1
D
V
DLK1
E
V
V
CD34
F
DLK1+ cells (relatively to number of fat cells)
perirenal
omental
pericardial
lower abdomen
tigh
breast
p < 0.001
vWAT
sWAT

The endothelium is consistently described as CD31$^+$ (Lin et al., 2010). The expression of the stem/progenitor cell marker CD34 by endothelial cells in small vessels in WAT is discussed controversial. As previously shown (Zimmerlin et al., 2010; Lin et al., 2010; Li et al., 2011), we detected two rings of CD34$^+$ cells, one in the outer adventitial stroma and another matching with the inner endothelium. In contrast to Lin et al. (2010), which described all endothelial cells positive for CD34, we identified both CD34$^-$ and CD34$^+$ vessels. This supports the hypothesis that well distinguishable subpopulations of CD34$^-$ and CD34$^+$ endothelial cells exists (Zimmerlin et al., 2010; Li et al., 2011). We found that the non-proliferative and non-adipogenic SVF fractions, cs-DLK1$^+$/cs-CD34$^-$ and cs-DLK$^+$/cs-CD34$^{+\,dim}$ cells, stained positive for CD31 in FACS analysis and we detected DLK1$^+$ cells in endothelial layers of small vessels in sWAT. The numbers of DLK1$^+$ cells in the endothelium differed between given vessels. Thus, it is conceivable that variants of DLK1$^+$ cells play a role in the endothelium while others exist as ASCs in vascular stroma. Similar to our data, the presence of DLK1$^+$ cells in adult endothelia was previously shown in adipose tissue of mice and DLK1$^+$/CD31$^+$ double-positive cells were identified in the SVF of mice WAT in the same study (Andersen et al., 2009a). Moreover, a more recent study showed that DLK1 can inhibit angiogenesis in vascular endothelium (Rodríguez et al., 2012). Thus, a role of a subset of DLK1$^+$ SVF cells in vascular endothelium of WAT is likely. In lineage tracing experiments in mice using DLK1 promoter activated expression of reporter genes to mark adipocyte progenitors, expression of endothelial markers was not detected in the labeled cells (Hudak et al., 2014) and neither endothelial nor hematopoietic lineage markers labeled adipocytes (Berry and Rodeheffer, 2013). Thus, it is possible that endothelial cells express DLK1$^+$ transiently during vessel growth and/or maturation.

The morphology of ASCs and the spatial organization of their niche surrounding small vessels in human WAT are not precisely known. We illustrated these cells in the context of small vessels in sWAT by TEM using DLK1 and CD34 as specific markers. Our ultrastructure analysis identified these ASCs in situ as cells with extremely long processes, showing a fibroblast-like morphology, similar to adipose progenitors described in previous studies (Cinti et al., 1984; Hausman et al., 1980), though DLK1$^+$ and CD34$^+$ cells were distinguishable from other stromal cells. DLK1$^+$ and CD34$^+$ cells preferentially resided in the adventitia of small blood vessels but also at capillaries and less frequently more peripherally between adipocytes. Our data indicate that the long processes of ASCs are very close to other cells in the microenvironment of WAT as described previously (reviewed in Hausman and Dodson, 2013). In fact, as a distinctive feature peripheral DLK1$^+$ ASCs appear to be very close or even in direct contact with adipocytes, which was especially verified by semi-thin sections and EM (Fig. 3D, N, and H). This spatial orientation of DLK1$^+$ ASCs raises the question whether these cells mark a population of further differentiating progenitors, which may form new adipocytes. Moreover, the long processes of DLK1$^+$ ASCs in the adventitia of small blood vessels were detected close to perivascular mural cells (Fig. 3I, M and I), which also form long processes (Berry et al., 2013; Cinti et al., 1984; Hausman and Dodson, 2013).

In adult mice, cells expressing the DLK1 gene exist in both sWAT and vWAT and DLK1 expressing cells are required for expansion of both WAT depot types in adult animals (Hudak et al., 2014). Our study indicates for the first time that the number of DLK1$^+$ cells in human vWAT is significantly lower than in sWAT depots. Given that DLK1 is a regulator of stem/progenitor cell fate (Andersen et al., 2009b, 2013; Ferrón et al., 2011; Hudak et al., 2014; Mortensen et al., 2012; Wang and Sul, 2009) and that in adipose tissues DLK1 is only found in adipocyte progenitors (Tang et al., 2008; Hudak et al., 2014), our data suggest that vWAT has a lower capacity to expand and regenerate than sWAT due to the presence of less DLK1$^+$ progenitor cells.

In conclusion, we detected different DLK1/CD34 subpopulations in sWAT. Only cs-DLK1$^-$/cs-CD34$^+$/CD90$^+$/CD105$^{+\,dim}$/α-SMA$^+$/CD45$^-$/CD31$^-$ cells exhibit proliferative and adipogenic capacity. These ASCs resemble mural cells with spindle-shaped morphology and long processes, they are however predominantly localized in the outer adventitial stromal ring of vessels and around capillaries. Significant numbers of DLK1$^+$ cells are also resident in vascular endothelium. Human vWAT contains less DLK1 + cells than sWAT.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.08.004.

Author disclosure statement

There are no potential conflicts of interest.

Acknowledgments

The work in the laboratory of H.F. was supported by intramural funding of the Medical University of Innsbruck. The work in the laboratory of W.Z. was supported by intramural funding of the University of Innsbruck.

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Fig. 6. Number of DLK1$^+$ cells in human visceral and subcutaneous WAT depots. Paraffin sections of omental (A), perirenal (B) and pericardial (C) vWAT and sWAT (D) depots from the same donors were stained using anti DLK1 antibodies. Representative photographs are shown. Specimens from 10 donors were analyzed and showed similar results. DLK1 positive cells are marked by arrow heads. (E) Consecutive paraffin section of the omental vWAT shown in (A) stained with anti-CD34 antibodies. V, vessel. Asterisk indicates the localization of the inserts. Bar: A–E (50 μm), Inset: A–E (5 μm). (F) Quantification of DLK1$^+$ cells in relation to fat cells in the given fat tissues, vWAT (perirenal, omental and pericardial) and sWAT (lower abdomen, thigh and breast). The mean value and the standard error of the mean (SEM) were specified. Data comparing vWAT and sWAT were analyzed by the Student's-t-test (two-tailed) for statistical significance, defining significance differences as P-values < 0.05, n = 10.

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